MOBILITY SHIFT OF BETA-DYSTROGLYCAN AS A MARKER OF *GMPPB* GENE RELATED MUSCULAR DYSTROPHY

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ABSTRACT

Background Defects in glycosylation of α-dystroglycan (α-DG) cause autosomal recessive disorders with wide clinical and genetic heterogeneity, with phenotypes ranging from congenital muscular dystrophies to milder limb girdle muscular dystrophies. Patients show variable reduction of immunoreactivity to antibodies specific for glyco-epitopes of α-DG on muscle biopsy. Recessive mutations in 18 genes, including GDP-mannose Pyrophosphorylase B (*GMPPB*), have been reported to date. With no specific clinical and pathological handles, diagnosis requires parallel or sequential analysis of all known genes.

Methods We describe clinical, genetic and biochemical findings of 21 patients with *GMPPB*-dystroglycanopathy.

Results We report 8 novel mutations and further expand current knowledge on clinical and muscle MRI features of this condition. In addition, we report a consistent shift in the mobility of β-DG on western blot analysis of all patients analysed by this mean. This was only observed in GMPPB patients in our large dystroglycanopathy cohort. We further demonstrate that this mobility shift in GMPPB patients was due to abnormal N-linked glycosylation of β-DG.

Conclusions Our data demonstrate that a change in β-DG electrophoretic mobility in patients with dystroglycanopathy is a distinctive marker of the molecular defect in *GMPPB.*

INTRODUCTION

Dystroglycanopathies are clinically and genetically heterogeneous muscular dystrophies caused by defective glycosylation of α-dystroglycan (α-DG) (1). Phenotypes range from severe congenital onset muscular dystrophies (CMD), often associated with brain and eye defects (such as Walker Warburg Syndrome and Muscle-eye-brain disease), to milder adult onset limb girdle muscular dystrophies (LGMD). The dystroglycan complex is encoded by the *DAG1* gene and translated as a propeptide proteolytically cleaved into α and β subunits. α-DG is an extracellular peripheral protein that undergoes extensive *N*- and *O*-linked glycosylation. The posttranslational modifications are essential for the interactions of α-DG with extracellular proteins, such as laminin-2 (*LAMA2*), nidogen, agrin, perlecan, neurexin, pikachurin and slit (1). β-DG is a transmembrane protein, with a single transmembrane region, three *N*-linked glycosylation sites, numerous potential phosphorylation sites and a proline enriched cytoplasmic tail. The dystroglycan subunits are non-covalently associated at the membrane and connect the extracellular matrix to the cytoskeleton acting as a cell adhesion receptor and as a player in cell adhesion-mediated signalling and cell polarity.

Primary dystroglycanopathies with mutations in *DAG1* gene remain extremely rare (2-4). On the other hand, the class of secondary dystroglycanopathies is ever-growing, with mutations in 18 genes reported to date (1,5). Suspicion of a dystroglycanopathy is based on clinical, brain imaging and pathological findings (6). Since mutations in all genes involved affect glycosylation modifications on α-DG, largely but not exclusively represented by *O*-linked glycans, muscle biopsies show alterations in labelling with antibodies against α-DG glyco-epitopes (such as IIH6 and VA4-1). The IIH6 epitope specifically recognises the G-domain binding site of α-DG

and indeed the use of blocking antibodies against IIH6 decouples this protein from laminin in cultured myoblasts (7). Quantitative or qualitative changes of β-DG immunolabelling are generally not present and reduction of laminin α2 may be observed (8). Diagnosis is established by identification of pathogenic mutations in one of the known dystroglycanopathy genes. Some of these genes (such as *FKTN* or *POMGnT1*) more commonly cause CMD, while others (in particular *FKRP*) show a wider phenotypic spectrum, with LGMD being the most prevalent phenotype. However, since no firm genotype-phenotype correlations have been demonstrated and there are no specific clinical or pathological markers to guide molecular investigations, the diagnosis must be achieved through the analysis of a large panel of genes.

Guanosine diphosphate mannose (GDP-mannose) pyrophosphorylase B (GMPPB), or mannose-1-phosphate guanyltransferase beta, is a cytoplasmic protein that catalyses the formation of GDP-mannose from mannose-1-phosphate and GTP. GDP-mannose is a key substrate for 4 distinct glycosylation pathways including *N*-glycosylation and *O*-mannosylation. Our group first described mutations in the *GMPPB* gene in patients with dystroglycanopathy (8). To date, 57 patients with *GMPPB* gene mutations have been reported. Phenotypes range from CMD to LGMD (LGMD2T), including isolated rhabdomyolysis and congenital myasthenic syndrome (CMS) with associated muscular dystrophy (9-19). Major inter and intrafamilial clinical variability is now recognised. Apart from the observation of neuromuscular junction abnormalities in a subset of patients with *GMPPB* gene mutations, no other findings distinguish this condition from other dystroglycanopathies.

Here we describe novel clinical, pathological and genetic findings on a large cohort of patients with *GMPPB* gene related muscular dystrophies (GMPPB-MD), further

expanding our knowledge on this emerging condition. A change in β-DG electrophoretic mobility was observed in the western blot of all biopsies analysed from this cohort, which was secondary to a defect in its N-glycosylation. This finding represents a novel and distinctive indicator of a molecular defect in the *GMPPB* gene and a biomarker for GMPPB-MD.

METHODS

Patients

This study was approved by the Health Research Authority, NRES Committee East of England – Hatfield (REC 13/EE/0398). Informed consent was obtained from patients and parents, where appropriate. Patients were selected among those referred to the Nationally Commissioned Highly Specialised Service (HSS) for CMD in London and LGMD in Newcastle upon Tyne with suspicion of dystroglycanopathy based on clinical, MRI or pathological ground. Reduction or absence of α-DG immunolabelling in patients who underwent muscle biopsy was the main criteria for activation of genetic analysis. In five patients (N8-12), genetic analysis of *GMPPB* was targeted prospectively following observation on the β-DG electrophoretic abnormality hereby reported. Clinical, muscle and brain MRI data, serum creatine kinase (CK) levels, neurophysiology and muscle pathology findings were obtained by retrospective analysis of clinical notes of patients included in the study. Cardiac function was investigated with echocardiogram and electrocardiogram. Respiratory function was assessed by annual spirometry. The cohort included 15 novel patients (N1-N14) and 6 previously reported individuals for whom we now report novel muscle MRI and protein data (patients 3, 7, 9-11 reported by Belaya et al., 2015; patient N1 by Carss et al., 2013) (8,12).

7

Genetic Analysis

Full gene sequencing of the *GMPPB* gene (Ref Sequence NM013334.2) was performed by Sanger sequencing and/or next generation panel sequencing at the Viapath Molecular Laboratory at Guys and St Thomas' Trust, London as part of the HSS for CMD (details available upon requests). In addition to the *GMPPB* gene, analysis of the *POMT1, POMT2, POMGNT1, FKRP, FKTN, LARGE, ISPD, GMPPB, B3GALNT2*, *B3GNT1, COL4A1, DAG1, POMK, DPM1, DPM2, DPM3*, *GTDC2* and *TMEM5* genes was also performed. *In silico* analysis of novel variants was performed using the Alamut mutation analysis software (Interactive Biosoftware, v1.5 http://www.interactivebiosoftware.com/). Novel variants were considered as likely pathogenic if affecting a moderate to highly conserved nucleotide, or a highly conserved residue, or if the resulting physiochemical difference between the wild-type and mutant amino acid was at least moderate. Affected and unaffected family members, where available, underwent segregation analysis to confirm phase of the variants identified and co-segregations with disease in the family.

Protein analysis

Protein analysis by immunohistochemistry and Multiplex Western blot was arranged with a battery of 35 diagnostic antibodies for LGMD, CMD and overlapping disorders including α-DG (IIH6, a kind gift of Dr. Kevin Campbell, University of Iowa, USA), β-DG and laminin-α2 (80 and 300 kDa fragments) as previously described (20- 21).

Deglycosylation Experiment

Proteins were extracted from muscle samples as previously described (22) and some incubated with N-Glycanase (Glyco, Prozyme) according to manufacturer's protocol. Immunoblotting of muscle protein lysates non-treated and treated was performed as previously described (22).

RESULTS

GMPPB gene mutations in novel GMPPB-MD patients

We identified 14 unrelated patients with pathogenic *GMPPB* gene mutations (Table 1). We identified 8 novel variants, one in homozygosity in 3 families. Six patients carried the recurrent p.Asp27His variant and two the p.Arg287Gln mutation.

Phenotypic analysis of novel GMPPB-MD patients

Five patients (N1, 3A-3B, 14-15) had a diagnosis of CMD while 8 presented with an LGMD phenotype (N2, 4-5, 8-12) (table 1). Patient N6, at age 13 years, had raised CK and intellectual disability (ID) with speech delay but no muscle weakness, while patient N7 at age 5 years only showed raised CK but completely normal intellect and no speech delay. Median age of onset in LGMD patients was mid-late 30s (range 1-49 years). All patients were ambulant at last assessment (median 31 years), and 11/15 were able to run. Pattern of muscle involvement was proximal>distal, affecting lower limbs more than upper limbs. Calf hypertrophy was noted in 4 patients. Variable degree of speech and learning delay was observed in 5 patients with CMD and 2 with LGMD . Three patients had microcephaly and one patient congenital cataract. One patient had ocular myasthenia. Neurophysiology showed myotonic discharges in two siblings (N3A-B). No patient had cardiac or respiratory insufficiency.

Muscle MRI imaging

Muscle MRI images were available for 5 novel patients (N2, 6, 10-12) and 2 previously reported cases (patient P1, Carrs et al; 2013 and Case 3, Belaya et al., 2015) (figure 1). MRI of patient N2 showed symmetric STIR signalling abnormality of gastrocnemious and soleus, and swelling of medial head of the gastrocnemius (figure 1A). A general reduction of thigh muscle bulk, with increase in muscle fat in

particular in the posterior compartment was found at age 9 in a CMD patient reported by Carrs et al, and the repeated scan 5 years later showed overall stable appearance (figure 1C and 1D). MRI scan of patient N6 showed very symmetric mild fatty streaking within both semitendinosus and sartorius muscles (figure 1B). Patients N10,N11, N12 and Case 3 of Belaya et al (2015) showed symmetrical posterior more than anterior involvement, more in upper than lower leg, atrophy of the rectus femoris, gastrocnemius (medial head more than lateral), tibialis anterior and peroneal muscles, with relative sparing of vastus intermedius, sartorius and gracilis muscles (figure 1E-1F and 1H). Spinal MRI in patient N9 showed selective atrophy of *erector spinae* and paraspinal muscles (data not shown).

Protein analysis

Muscle biopsies of all patients displayed a variable range of myopathic or dystrophic changes and patchy expression of α-DG. Laminin-α2 showed mild reduction of labelling in some but not in all cases. Immunolabelling of β-DG was normal in all patients on sections (data not shown). Western blot analysis consistently showed a slightly higher mobility (reflected as a slightly lower molecular mass) of β-DG in 12/12 patients GMPPB samples analysed, often seen together with a reduction of laminin-α2 (80 kDa fragment) (figure 2A and 2B). The mobility shift of β-DG was not observed in samples from patients with dystroglycanopathy caused by mutations in other genes including *FKRP* (34 patients), *POMGnT1* (3 patients), *POMT1* (2 patients), *POMT2* (2 patients), *ISPD* (2 patients) and *B3GALNT2* (2 patients; see figure 2B and not shown). β-DG harbors 3 N-linked glycosylation sites. Since the conversion of mannose-1-phosphate and GTP to GDP-mannose catalyzed by GMPPB is a common step for both O- and N-linked glycosylation, we proceeded to assess whether the mobility shift of β-DG was due to a defect in N-glycosylation. N-

glycanase treated muscle tissue of healthy control and GMPPB-MD patients showed similar electrophoretic mobility (figure 2C). β-DG molecular mobility was increased in N-glycanase treated GMPPB samples compared to untreated ones (supplemental figure 1). These results suggest that the observed mobility shift in GMPPB-MD patients is secondary to the defective Dystroglycan N-glycosylation.

DISCUSSION

Mutations in the *GMPPB* gene cause a broad spectrum of increasingly prevalent dystroglycanopathies. Phenotypic analysis of the families reported here confirms and further expands our knowledge of *GMPPB* associated phenotypes. In particular, our data confirms the prevalence of LGMD phenotype in association with *GMPPB* gene mutations and expands the phenotypic spectrum by describing a patient with mental retardation and raised CK only at age 13 years. We confirm a high frequency of learning and speech delay, relatively high occurrence of microcephaly in CMD patients but lack of major cardiac and respiratory involvement. The cause and significance of the myotonic discharges observed in the 2 siblings from a consanguineous family is unclear. However, as this has never been reported in patients with *GMPPB* gene mutations before, it might represent an additional uncharacterised condition present in this consanguineous family. Patterns of muscle involvement on MRI in LGMD patients were strikingly similar, with symmetrical posterior>anterior involvement and specific muscle atrophies in particular of *erector spinae*, *rectus femoris* and peroneal muscles. This pattern is similar to that reported by Oestergaard et al. (16), and partly resembles patterns observed in LGMD2A and to a lesser degree LGMD2I (23). We also observed no/minimal muscle MRI involvement in patients with milder muscle phenotype.

Including this cohort, *GMPPB* gene mutations have so far been described in 72 patients. Review of clinical data for published patients evidenced LGMD as the most common phenotypic presentation of *GMPPB*-DG (42 patients, 58%). CMD (18 patients, 25%), and CMS-like conditions (8 patients, 11%) are less common, although clinical and electrophysiological features of CMS were also reported in 12 LGMD patients (27%). Milder phenotypes, such as isolated ID, rhabdomyolysis, metabolic myopathy or isolated hyperCKaemia were observed in 4 individuals, although these patients could develop further symptoms with age. Among CMD patients, CMD-MR was the most common subtype (9/18 patients), but CMD-CRB and CMD-MEB were also reported. Average age at onset in LGMD and in CMS was late teens and early 20s, respectively. A variable degree of learning difficulties was observed in patients with any type of presentations, including 23% of LGMD patients and great majority of CMD patients, and in at least 3 patients this represented the main clinical feature, in association with no or fairly minimal muscle weakness at age 13, 14 and 36 years and CK in the range of 3000-4000 IU/L (present cohort; 10 and 12). Overall, these data show that *GMPPB* mutations are more likely to cause LGMD or LGMD/CMS overlap syndromes rather than congenital onset conditions and some degree of ID is a frequent observation. Considering that all cohorts so far come from groups with predominant expertise in muscular dystrophies, the observation that hyperCKaemia, isolated or associated with rhabdomyolysis or ID, can be predominant features in *GMPPB* related conditions suggest that the prevalence of this condition could be higher if these unusual phenotypes were systematically studied for the involvement of this gene.

A total of 42 *GMPPB* mutations are known to date, all but 7 being missense changes. Two variants are more common, with the p.Asp27His and p.Arg287Gln mutations

representing 24% and 10% of all alleles. Of note, a further 7% of alleles also affected residue 287 (c.859C>T p.(Arg287Trp)), but mostly found in the LGMD patients (5/7 patients). Our analysis indicates additional hotspots, such as at residue Pro32, Pro103 and Arg185. We have reported a novel in frame 4 nucleotide deletion, occurring in homozygosity in 3 apparently unrelated families, leading to deletion of residue 270 and substitution in position 271. Interestingly, mutations affecting residues between Pro241 to Asn271 were also frequent. Previous reports indicated some genotype-phenotype correlations, with the two common variants p.Asp27His and p.Arg287Gln being associated with LGMD and CMD, respectively. Our data further confirm this finding, with the p.Asp27His mutation being only present in LGMD or asymptomatic patients from our cohort (table 1). The distribution of mutations does not appear to correlate with clinical severity and we did not identify any genotype phenotype correlation with nonsense/splice site mutations.

Dystroglycanopathies are mostly diagnosed based on the depletion of IIH6 immunostaining but no pathology biomarkers specific for each genetic subtype is known. The increasing knowledge of the prevalence of specific genetic subtypes and their association with variable clinical presentations helps with targeted gene analysis (24-25). However, the ever-increasing number of genes associated with dystroglycanopathies makes genetic diagnosis rather challenging and despite major advances, parallel gene sequencing of all known genes is still the most validated diagnostic approach. Furthermore, a number of patients remain without genetic confirmation and novel genes might be more common than expected. We have now identified a novel and distinctive marker, a β-DG mobility shift on WB that, in combination to abnormal labelling of IIH6, can guide further genetic analysis of dystroglycanopathies. Our data showed 100% specificity and sensitivity, as all

13

patients with this biomarker had a diagnosis of GMPPB-MD; while this was a
universal feature in GMPPB patients, the shift was more evident in some patients than
others, and did not correlate with age. Of note, 5 patients were identified based on
retrospective review of WB analysis of genetically undiagnosed dystroglycanopathy
patients and observation of the typical β-DG mobility. We have not identified a
similar mobility shift before in analysis of 45 patients with at least 6 genetically
proven dystroglycanopathies (figure 2; 26-27). Furthermore, we did not see the
mobility shift in additional genetically undiagnosed dystroglycanopathy patients
tested with our gene panel. In view of this, we suggest that this β-DG mobility shift in
combination with abnormal immunolabelling of IIH6 could be considered a strong
indicator for GMPPB-MD, which can therefore be used to directly guide the genetic
diagnosis. WB analysis of patient fibroblasts from this study and from Carss et al. (8)
showed that β-DG was normal compared to controls (data not shown) suggesting that
the posttranslational event affected by mutations in the *GMPPB* gene may be tissue
specific. GMPPB is a key enzyme for both *N-* and *O-* glycosylation. Therefore we
hypothesised that the observed mobility shift of β-DG was due to abnormal N-linked
glycosylation by means of a defective GMPPB protein. This was confirmed by
observing deglycosylated bands of identical size in both controls and patients after N-
glycanase treatment (figure 2C). GMPPB catalyzes the synthesis of GDP-mannose
from GTP and mannose-1-phosphate. GDP-mannose is the substrate of cytosolic
mannosyltransferases required for the synthesis of the core N-glycan structure, and it
is required for the synthesis of Dol-P-Man in the ER membrane. Dol-P-Man synthesis
is catalyzed by the DPM synthase complex, consisting of DPM1, DPM2, and DPM3.
Dol-P-Man is the mannose donor required for all four mannosylation reactions that
occur in the ER: O-mannosylation, C-mannosylation, N-glycosylation, and

14

glycosylphosphatidylinositol-anchor formation. As ß-Dystroglycan contains three N-linked glycosylation site (28), we infer that the deficiency of GMPPB results in defective N-glycosylation of this crucial muscle protein. Of note, patients with GMPPB gene mutations, when tested, showed normal routine diagnostic screening for CDG (8).

In conclusion, this is the first report of a biochemical marker of a specific genetic type of dystroglycanopathies. Importantly, we demonstrate that mutations in *GMPPB* affect glycosylation of α as well of β-DG. Detection of changes in β-DG electrophoretic mobility in muscle biopsies has the potential to refine the laboratory's diagnostic capabilities and ultimately improve clinical care. The defective N-glycosylation of β-DG could potentially contribute to some of the unique features observed in GMPPB patients, including defective neuromuscular junction signal transmission, but this will require further studies.

A. Contributorship

AS drafted the manuscript, reviewed clinical and MRI data and contributed to plan the study, ST planned the study and performed laboratory investigations; RM performed genetic testing; MH performed protein analysis; RP, LH and CS performed histopathological investigations and critical review of muscle biopsy data; PA performed laboratory investigations; MS collected and analysed clinical data; MY performed genetic testing; MB, TW, SH, AM, FN, WR, AR, SSV, MP, MEF, CO, GM, CMB, EW, DM, HL, VS, KB collected and clinically reviewed clinical and MRI data; MW, SM conducted further histopathogical investigations; RB designed the study, critically reviewed results, content of the work and manuscript as guarantor of the study; FM has reviewed the results, manuscript and is responsible for the overall content as guarantor.

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Figure Legends

Figure 1: Muscle MRI images of patients with *GMPPB* gene mutations.

Images A, E,F and H show the pattern of muscle involvement in 4 patients with LGMD (Pt N2, 10,11 and 12). Note the symmetrical posterior >anterior involvement in upper legs, with atrophy of the rectus femoris with sparing of vastus intermedius, sartorius and gracilis muscles in E, F and H. A variable degree of atrophy of the gastrocnemius tibialis anterior and peroneal muscles is noted in the lower legs (E,F and H). Image 1G shows a similar pattern of muscle involvement in a CMS patient with LGMD weakness (Case 3 Belaya et al 2015). Symmetrical mild fatty streaking in semitendinosus and sartorius muscles is noted in image B relative to patient N6 with ID. Reduction of thigh muscle bulk and increase in muscle fat in the posterior compartment is evidenced in 2 sequential studies for at age 9 (D) and 14 years (E) for a CMD patient (Patient P1, Carss et al, 2013).

Figure 2: Western blot analysis of patients with *GMPPB* gene mutations and other genetically proven dystroglycanopathies

A) Consistent lower mobility shift of β-DG in 6 patients with *GMPPB* gene mutations. B) The shift is not observed in samples from 2 patients with mutations in *FKRP* and one patient with mutations in *POMGnT1* gene. C) Bands of equal size are observed in control and patients after N-Glycanase treatment.

**Supplemental Figure 1: Western blot analysis of N-glycanase treated muscle tissue of control and GMPPB-MD patients**

Lane 1-4: Western blot analysis of muscle tissue of healthy control and patients without N-glycanase treatment (-); lane 5-8: Western blot analysis of muscle tissue of healthy control and patients after N-glycanase treatment (+).

## Table 1: Clinical and genetic characteristics of 15 novel patients

Pt	Sex	Age	Phenotype	onset (years)	CK (max)	Symptoms at onset	Main muscle finding	Max ability	Cognitive function	EMG	Further clinical findings	Mutation1	Mutation 2
N1	F	7	CMD-MR	0	3300	Speech/motor delay, microcephaly, CK↑	Generalised weakness, TA tightness	Walk	Intellectual + speech delay	N	MC, feeding difficulties	**c.810_813delinsTGGC p.(Asn271Gly)**	**c.810_813delinsTGGC p.(Asn271Gly)**
N2	F	14	LGMD	1	2900	Developmental delay	Mild proximal weakness, easy fatigue, calf hypertrophy	Walk	Intellectual delay	N		**c.841G>A p.(Glu281Gln)**	c.860G>A p.(Arg287Gln)
N3 A	F	5	CMD	0	770	Developmental delay, cataract	Generalised weakness	Run	Intellectual delay	Myotonic discharges	Congenital cataract, low weight, recurrent chest infections, hypermobility	**c.810_813delinsTGGC p.(Asn271Gly)**	**c.810_813delinsTGGC p.(Asn271Gly)**
N3 B	M	7	CMD	0	1300	Developmental delay	Generalised weakness	Run	Intellectual delay, ADHD	Myotonic discharges	MC, feeding difficulties, hypermobility	**c.810_813delinsTGGC p.(Asn271Gly)**	**c.810_813delinsTGGC p.(Asn271Gly)**
N4	M	55	LGMD	17	3100	Fatigue	Proximal weakness	Run	N	Myopathic		**c.727C>T p.(Arg243Trp)**	**c.754G>T p.(Gly252Cys)**
N5	F	53	LGMD	20	2300	Proximal weakness	LL proximal weakness, scapular winging, calf hypertrophy	Run	N	NA		c.79G>C p.(Asp27His)	c.859C>T p.(Arg287Trp)
N6	F	13	MR	4	4000	Speech delay, toe-walking, CK↑	Bilateral talipes	Run	Intellectual + speech delay	N		c.1000G>A p.(Asp334Asn)	c.1000G>A p.(Asp334Asn)
N7	M	5	-	-	1200	-	N	Run	N	NA		c.79G>A p.(Asp27His)	c. 988G>A p.(Val330Ile)
N8	M	72	LGMD	49	1200	Proximal weakness	Proximal weakness, scapular winging, calf hypertrophy, lumbar rigidity, fatigue	Walk	N	Myopathic		**c.338G>A p.(Cys113Tyr)**	c.860G>A p.(Arg287Gln)

ID	Sex	Age	Diagnosis		CK	Presenting symptom	Distribution		Cognition	EMG	Other	Mutation 1	Mutation 2
N9	M	69	LGMD	48	4000	Proximal weakness	LL> UL Proximal weakness, scapular winging	Run	N	NA		c.79G>A p.(Asp27His)	**c.87C>A p.(Cys29Ter)**
N10	M	45	LGMD	43	1300	Proximal weakness	Axial and LL proximal weakness, calf atrophy	Run	N	Myopathic mild decrement	Back pain	c.79G>C p.(Asp27His)	c.95C>T p.(Pro32Leu)
N11	F	58	LGMD	35	780	Proximal weakness	Axial, proximal> distal weakness, calf hypertrophy	Walk	N	Myopathic membrane instability		c.79G>A p.(Asp27His)	c.907C>T p.(Leu303Phe)
N12	F	63	LGMD	43	3800	Myalgia, falls, stiffness	Axial, proximal>distal weakness, stiffness, foot drop, feet and ankle deformities	Run	N	Myopathic	Ocular myasthenia gravis	c.79G>C p.(Asp27His)	**c.1108G>C p.(Val370Leu)**
N13	M	8	CMD	0	1000	Developmental delay, raised CK		Run	Intellectual +speech delay	NA	MC	**c.810_813delinsTGGC p.(Asn271Gly)**	**c.810_813delinsTGGC p.(Asn271Gly)**
N14	M	6	CMD	0.4	3200	Developmental delay	Fatigue, mild generalised weakness, more prominent axially	Run	Intellectual + speech delay	NA	Torticollis, atrial septal defect	c.1069G>A p.(Val357Ile)	**c.1081G>A p.(Asp361Asn)**

Legend: CK creatine kinase, F female, M male, CMD Congenital muscular dystrophy, , LGMD limb girdle muscular dystrophy, TA Achilles tendons, MC, microcephaly, N normal, NA not available, LL lower limbs, UL upper limbs, > more. Novel mutations are indicated in bold.
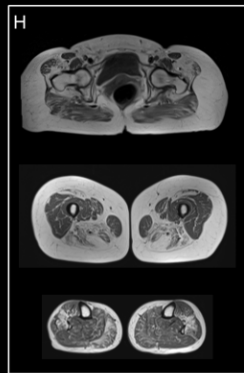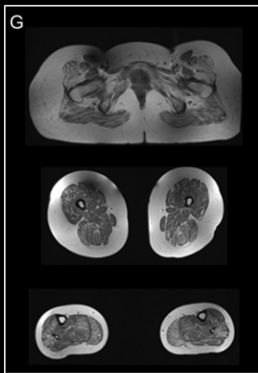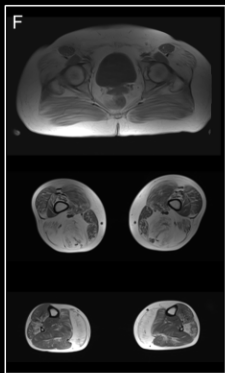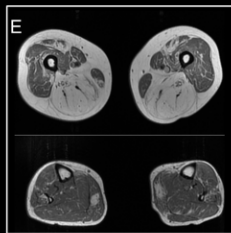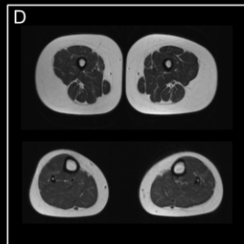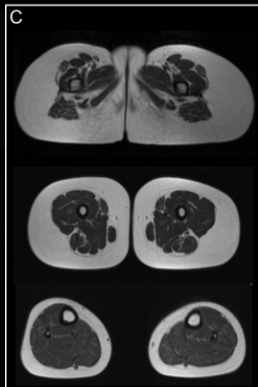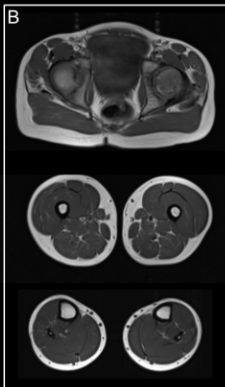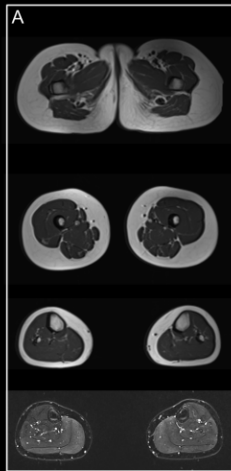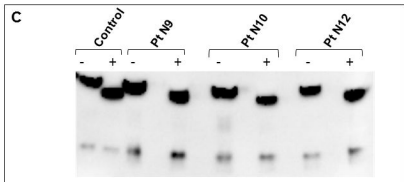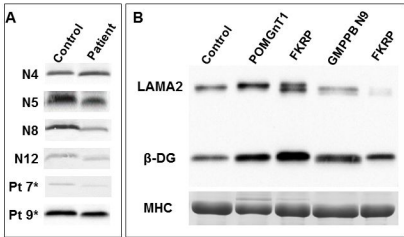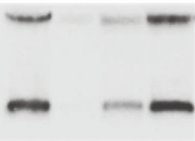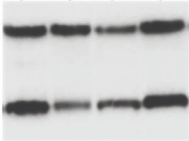
C	N12	N10	N9	C	N12	N10	N9	N-glycase
−	−	−	−	+	+	+	+	

43kDa

30kDa